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Packaged replicons of bovine viral diarrhea virus are capable of inducing a protective immune response

Ilona Reimann [a,1], Ilia Semmler [b,1,2], Martin Beer [b,*]

[a] Institute of Molecular Biology, Friedrich-Loeffler-Institut, Boddenblick 5a, 17493 Greifswald-Insel Riems, Germany
[b] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Boddenblick 5a, 17493 Greifswald-Insel Riems, Germany

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Abstract

Bovine viral diarrhea virus (BVDV) replicons with deletions within the capsid, E^{RNS} or E1 encoding region were constructed and efficiently packaged with a helper cell line. High titres of packaged replicons were observed as early as 24 h after transfection, whereas no virus progeny could be detected after transfection of non-complementing cells. Infection of bovine cell cultures with rescued viruses resulted in one cycle of replication without release of infectious virus particles, and no genetic reversion of the generated viruses was detected. Packaged replicons with a deletion within the capsid-coding region were characterized *in vivo* in immunization and challenge trials. Following immunization of calves with the replication-deficient virus, neither virus shedding nor viremia was detected. After challenge infection with virulent BVDV, all vaccinates were completely protected from disease as measured by the absence of viremia and shedding of challenge virus, which indicated that a 'sterilizing immunity' could be induced with the generated replication-deficient packaged replicons.

Keywords: Bovine viral diarrhea virus; *Pestivirus*; *Trans*-complementation; Replicon; Vaccine

Introduction

The family *Flaviviridae* contains a number of important human and animal pathogens which are classified into three closely related genera: *Flavivirus* within which for example tick-borne encephalitis virus (TBEV), West Nile virus (WNV), yellow fever virus (YFV) and kunjin virus (KUN) are grouped, *Hepacivirus* represented by hepatitis C virus (HCV), and finally *Pestivirus*, which represents four different species: bovine viral diarrhea virus type 1 and 2 (BVDV-1, BVDV-2), classical swine fever virus (CSFV) and border disease virus (BDV) (Fauquet et al., 2005). HCV is responsible for severe chronic liver disease in humans (Overby et al., 1989), but no efficacious vaccine against the infection has become available.

Pestiviruses can be divided into two different biotypes, cytopathogenic (cp) and non-cytopathogenic (ncp) viruses, respectively. BVDV is the causative agent of bovine viral diarrhea, an economically important disease worldwide (Brownlie et al., 1984; Houe, 1995). The major disease manifestations of BVDV infections are located to the genital and reproductive organs and infections result in reduced fertility, abortions and the generation of persistently infected calves, which can develop fatal "Mucosal Disease" (Baker, 1995; Bolin, 1995a; Moennig and Liess, 1995). The pestivirus genome is a single-stranded RNA of positive orientation with a length of approximately 12.3 kb. It contains one large open reading frame (ORF), which is flanked by non-translated regions (NTR) at both genome termini. The large ORF is translated into one polyprotein, which is co- and post-translationally processed into 12 mature proteins by viral and cellular proteases (Collett et al., 1988; Thiel et al., 1991; Rümenapf et al., 1993). In cp BVDV, nonstructural protein NS3 is more abundantly expressed due to variable insertions allowing a more efficient cleavage of nonstructural proteins NS2 and NS3 (Collett et al., 1989; Lackner et al., 2004, 2005). *Pestivirus*

* Corresponding author. Fax: +49 38351 7 151.
E-mail address: martin.beer@fli.bund.de (M. Beer).
[1] Ilona Reimann and Ilia Semmler contributed equally to the study.
[2] Present address: Robert Koch-Institute, Nordufer 20, 13353 Berlin, Germany.

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particles consist of four structural proteins, the capsid protein C and three glycosylated envelope proteins (E^{RNS}, E1, and E2). In natural infection, BVDV antibodies are directed against E^{RNS}, E2 and NS3, but neutralizing activity was predominantly demonstrated for E2-specific antibodies (Donis, 1995; Potgieter, 1995).

Pestivirus genomes with deletions were first described as naturally occurring, defective interfering particles (DIs) for BVDV and CSFV (Kupfermann et al., 1996; Meyers and Thiel, 1995). Many reports relating to the replication of pestiviruses have been published since the discovery of DIs, the construction of a host of mutants now considerably facilitated by reverse genetics systems (Donis, 1995; Kupfermann et al., 1996; Meyers et al., 1996, 2002; Moormann et al., 1996; Rüggli et al., 1996; Tratschin et al., 1998; Kümmerer and Meyers, 2000). The development of autonomously replicating RNAs (replicons) was the first step towards establishing a cell culture-based replication system for HCV (Lindenbach et al., 2005; Lohmann et al., 1999), and chimeric HCV, which could be passaged efficiently in cultured cells, have recently been reported (Lindenbach et al., 2005). The number of cell types permissive for HCV replication is limited, however, and no packaging system has been reported. In contrast, replicons derived from other flaviviruses have been shown to be packaged with the help of complementing cell lines (reviewed in Mandl, 2004; Pijlman et al., 2006a; Schlesinger and Dubensky, 1999), as was shown for YFV (Lindenbach and Rice, 1997), KUN (Harvey et al., 2004; Khromykh et al., 1998, 1999; Liu et al., 2002; Pijlman et al., 2006b), WNV (Scholle et al., 2004) or TBEV (Gehrke et al., 2003, 2005). In the case of CSFV, *trans*-complemented defective virions that contained either E^{RNS} or E2 deleted replicons were shown to potentially serve as vaccine candidates (Maurer et al., 2005; van Gennip et al., 2002; Widjojoatmodjo et al., 2000). These replicons were *trans*-complemented using cell lines constitutively expressing the structural proteins previously deleted in the replicon genomes. In animal experiments, pigs were partially protected against lethal CSFV challenge after immunization with the complemented virions, and the reported efficacy of packaged CSFV replicons was dependent on the used deletion mutant as well as the way of application, with the best results obtained after intradermal administration (van Gennip et al., 2002).

Only few experiments on *trans*-complementation of BVDV replicons have been reported. Defects in the coding region for nonstructural proteins NS3, NS4a, NS4B and NS5B were found to be lethal and could not be complemented *in trans*, whereas genomic defects in NS5A could be complemented (Grassmann et al., 2001). Chimeric BVDV in which genes encoding capsid, E^{RNS}, E1 and E2 were replaced by its HCV counterparts, could be packaged into virions containing the BVDV structural proteins provided by a helper virus (Nam et al., 2001). *Trans*-complementation of BVDV E2 and p7 has also been described (Harada et al., 2000), and Reimann et al. (2003) demonstrated that autonomously replicating E2 deletion mutants were efficiently *trans*-complemented using a helper cell line (PT_805) providing all structural BVDV proteins.

Here we describe the construction and *trans*-complementation of BVDV genomes with deletions in the C, E^{RNS} or E1 encoding region, respectively. All BVDV deletion mutants replicated autonomously, but failed to generate infectious virus progeny in non-complementing cells. Nevertheless, the different deletion mutants could be efficiently packaged using a helper cell line, and packaged replicons devoid of capsid ('pseudovirions') were shown to induce a sterile immunity. Novel BVDV replicons are therefore considered highly efficacious and safe vaccine candidates.

Results

Construction and characterization of BVDV replicons

We constructed a set of BVDV replicons based on a full-length infectious cDNA clone of non-cytopathogenic BVDV strain NCP7 (pA/BVDV/INS^{-}; Meyers et al., 1996). Partial deletions were introduced in regions coding for a single structural protein: capsid (NCP7ΔC), E^{RNS} (NCP7Δ E^{RNS}) or E1 (NCP7Δ E1) (Fig. 1). The different replicons contained deletions of amino acids 201 to 242 (NCP7ΔC), 321 to 475 (NCP7ΔE^{RNS}), or 498 to 654 (NCP7ΔE1).

RNA transcribed from the plasmids encoding NCP7ΔC, NCP7ΔE^{RNS} or NCP7ΔE1 all efficiently replicated in bovine cell culture post transfection (p.t.). Expression of NS2/3 could be detected at 24 h p.t. by IF staining for all newly synthesized replicon RNAs. The intensity of NS2/3-specific immunostaining of NCP7ΔC-, NCP7ΔE^{RNS}- or NCP7ΔE1-transfected PT cells was comparable to cells transfected with full-length NCP7 RNA, and more than 85% of the cells were IF-positive (Table 1). No RNA replication could be detected after transfection of replication-defective NCP7Δ3′AatII RNA (Reimann et al., 2003), which was used as a negative control. Transfection of full-length NCP7 RNA into non-complementing PT cells resulted in virus titers of up to 10^3 IU/ml at 24 h p.t. In contrast, no infectious recombinant BVDV could be recovered after transfection of the deletion mutants, and all inoculated KOP-R cell cultures, which are highly susceptible to BVDV infection (Reimann et al., 2004), remained negative even after serial passages and co-passages. However, after reinsertion of the genes coding for C, E^{RNS} or E1 into the respective mutant replicons, growth of each of the constructed revertant viruses was indistinguishable from original BVDV NCP7 (data not shown).

Recovery and characterization of recombinant BVDV complemented in trans

In order to generate *trans*-complemented virus, *in vitro*-transcribed RNA of the NCP7ΔC, NCP7ΔE^{RNS}, NCP7ΔE1, NCP7Δ3′AatII or full-length NCP7 was transfected into the complementing helper cell line PT_805 that constitutively expresses BVDV structural proteins (Reimann et al., 2003). At 24 h p.t., monolayers of PT_805 cells were examined for NS2/3 expression and culture supernatants were passaged and titrated using KOP-R cells. For all replicons and full-length clone NCP7, NS2/3 staining following RNA transfection led to immunofluorescence patterns similar to that obtained with parental, non-complementing PT cells. Between 50% and 95%

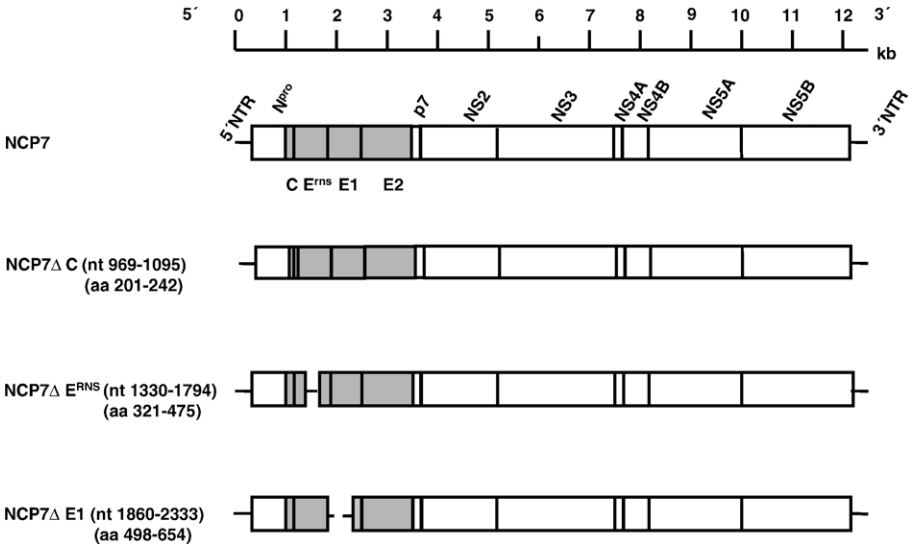

Fig. 1. Schematic diagram of constructs generated in this study and compared to parental full-length BVDV NCP7. Filled boxes represent the BVDV structural protein region. Horizontal dotted lines show the deleted regions and numbers indicate the nucleotide (nt) or amino acid (aa) position in the BVDV full-length RNA. Lines at the left and the right ends indicate untranslated regions. N^{pro}, autoprotease; C, capsid protein; E^{RNS}, E1, E2, envelope proteins; p7, nonstructural protein; NS2 to NS5, nonstuctural proteins; 3′NTR and 5′NTR, non-coding regions. The scale in kb is given.

of the transfected cells reacted with NS3-specific antibodies (data not shown). Infectious, recombinant BVDV was detected, however, only in supernatants from PT_805 cells transfected with NCP7ΔC, NCP7ΔE^{RNS}, NCP7ΔE1 or NCP7 RNA (Table 1). Inoculation of KOP-R cells with the packaged replicons (so-called 'pseudovirions') present in the PT_805 supernatants resulted in positive IF staining, and it was evident that only single cells or two adjacent cells, likely in the process of cell division, were expressing BVDV proteins (Figs. 2A and B). In addition, the pseudovirions were not able to spread from cell to cell to form foci typical for replicating and spreading BVDV (Fig. 2B). Virus titers of the complemented viruses NCP7ΔC_trans, NCP7ΔE1_trans and NCP7ΔE^{RNS}_trans varied between $10^{5.5}$ and $10^{6.125}$ IU/ml at 24 h p.t. (Table 1). After transfection of full-length NCP7 RNA into complementing PT_805 cells, virus titers of $10^{7.3}$ IU/ml could be detected at that time point. In contrast, no complemented BVDV could be detected after passaging of PT_805 cell-culture supernatants transfected with replication defective NCP7Δ3′AatII (Table 1). Furthermore, PT_805 cells were more efficient in packaging of the replicons than alternative helper systems, like another cell line (PT_875) expressing only BVDV capsid and E^{RNS} or Sindbis virus replicon-expressed proteins (data not shown).

Cell cultures inoculated with the different types of pseudovirions were stained with E2-, NS3- and E^{RNS}-specific monoclonal antibodies. Cells infected with NCP7-, NCP7ΔC_trans or NCP7ΔE1_trans could specifically be stained using the whole set of Mabs at our disposition, while cells infected with NCP7ΔE^{RNS}_trans, as expected, remained negative after staining with BVDV E^{RNS}-specific antibodies (Fig. 2A). The identity

Table 1
Replication and *trans*-complementation of BVDV replicons

Construct	Replication[a] after RNA transfection in PT cells (24 h)	1st passage[b] (24 h, 48 h, 72 h) on KOP-R-cells	Titer of progeny virus[c] 24 h after transfection of complementing PT_805 cells (IU/ml)	4th passage[d] of virus progeny on KOP-R cells
NCP7	++ (P)[e]	+ (10^3 IU/ml)	$10^{7.3}$ (P)	+ (P)
NCP7ΔC	++[f]	∅[f]	$10^{6.125}$	∅
NCP7ΔE^{RNS}	++	∅	$10^{6.00}$	∅
NCP7ΔE1	++	∅	$10^{5.50}$	∅
NCP7ΔAatII	∅	∅	$<10^0$	nd[g]

[a] PT cells were transfected with *in vitro*-transcribed RNA and IF-stained for NS3-expression after 24 h.

[b] KOP-R cells were inoculated with undiluted supernatants 24 h, 48 h and 72 h after transfection of PT cells (1 ml per 10^5 cells). KOP-R cells were stained with NS3-specific Mabs 5 days post inoculation.

[c] PT_805 cells were transfected with *in vitro*-transcribed RNA and the supernatants were titrated after 24 h using KOP-R-cells. The maximum titer of more than three independent experiments is shown as infectious units (IU; Reimann et al., 2003).

[d] KOP-R cells were inoculated using supernatants of transfected PT_805 cells with a titer $>10^5$ IU/ml (1 ml per 10^5 cells). The results of more than 10 different inoculation experiments are shown.

[e] P indicates the detection of virus plaques after NS3-staining (>10 cells).

[f] ∅=no BVDV NS3-specific IF-signal; +=weak positive NS3-IF-signal and <50% IF-positive cells, ++=positive NS3-IF-signal and >50% IF-positive cells.

[g] nd=not done.

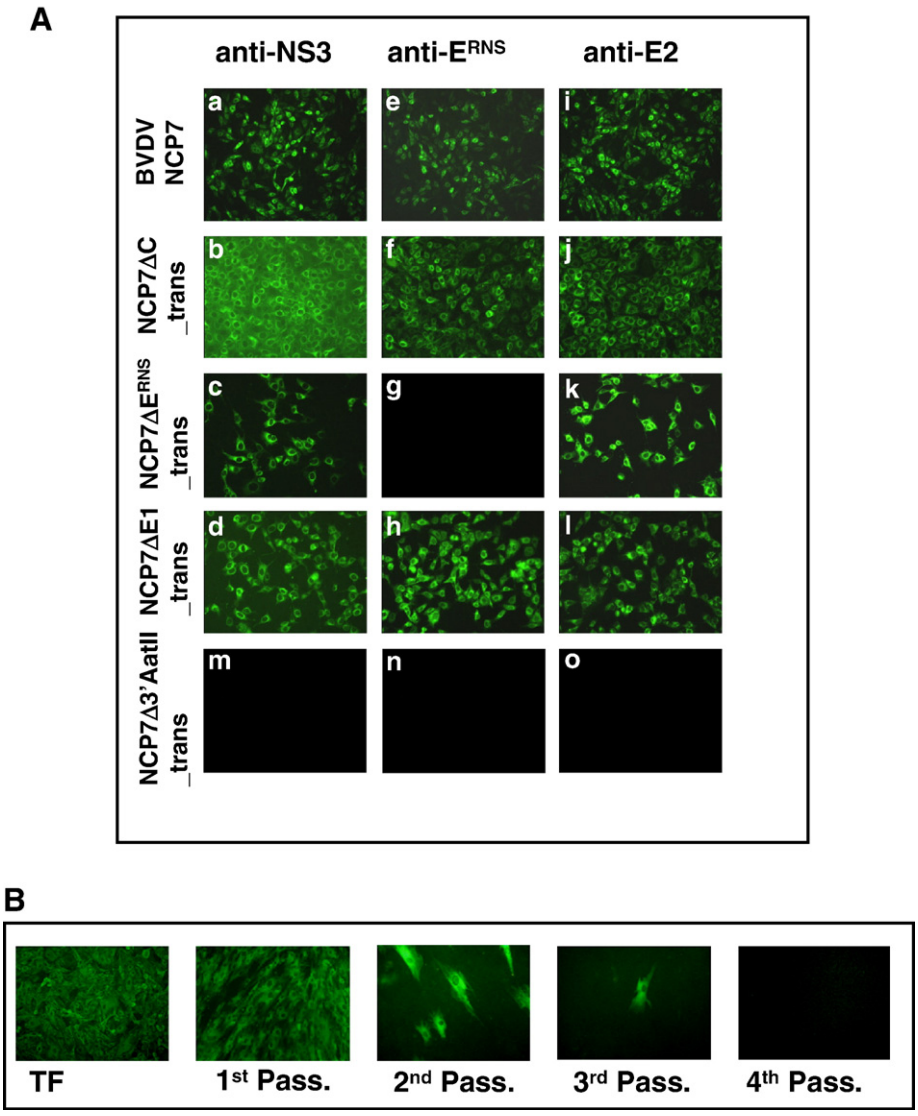

Fig. 2. (A) IF analysis of KOP-R cell cultures inoculated with trans-complemented replicons. Inoculated cells were analysed 24 h p.i. by IF using NS3-, E^{RNS}-, or E2-specific monoclonal antibodies (C16, WB210, CA3). Infectious virus could be detected in supernatants of PT_805 cells transfected with all of the engineered replicon RNAs or RNA of the full-length NCP7 (a–l), but not from cells infected with replication-deficient NCP7Δ3′AatII (m–o). Expression of E^{RNS} could not be detected in cells inoculated with supernatants from packaged NCP7ΔE^{RNS} replicons (g). (B) Serial passage of packaged NCP7ΔC replicons on non-complementing bovine KOP-R cells. Transfected (TF) cells as well as cells infected with supernatants of the various passages were detected by immunofluorescent staining for BVDV non-structural protein NS3 using monoclonal antibody C16. Only single infected cells were detectable after the 2nd passage, and, following the 4th serial passage, all cells were negative for NS3 expression.

of the packaged RNAs with a deletion within the capsid- or E1-encoding regions were additionally analysed by RT–PCR using RNA extracted from bovine cells infected with the packaged replicons. Two primer pairs matching the capsid, E^{RNS} or E1 sequences of NCP7 or the heterologous sequence of the complementing cell line PT_805 were also used to exclude recombinations or reversions of the deleted genomes. Using highly sensitive RT–PCR analyses we could confirm not only the identity of all preparations of packaged replicons but also the absence of revertants or pseudo-revertants in all preparations of pseudovirions (data not shown). Consecutive passages using non-complementing cells also demonstrated that the packaged replicons were capable of replicating in bovine cells without generating infectious virus progeny, as is exemplarily shown for 4 consecutive passages of NCP7ΔC_trans in KOP-R cells (Fig. 2B).

Immunization and challenge infection of cattle with NCP7ΔC_trans pseudovirions

After immunization of calves with NCP7ΔC_trans pseudovirions, the general condition of the animals remained unaltered, irrespective of the immunization route, and neither clinical signs nor shedding of pseudovirions, viremia, or leukopenia were observed. In addition, virus isolation from blood leukocytes after immunization was negative for all animals (data not shown). Immunized animals in all groups developed NS3-specific antibodies following the first immunization with inhibition

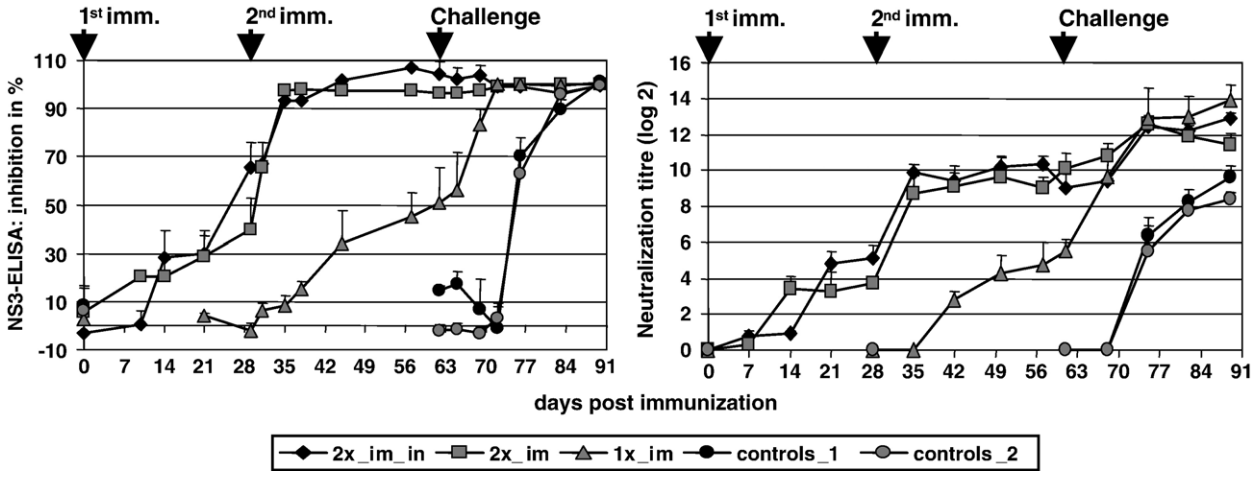

Fig. 3. Serological responses (NS3-specific antibodies as well as neutralizing antibody titers) after immunization and challenge infection are shown as means and standard deviations (error bars) for the individual groups.

values of 40% to 65%, which increased to more than 90% after the second immunization (Fig. 3). The neutralizing antibody titers against the homologous BVDV strain NCP7 (Fig. 3) and the heterologous BVDV challenge strain SE5508 (data not shown) ranged from 1:16 to 1:32 after the first immunization, which increased to values of greater than 1:256 after booster vaccination. No marked differences between the immunization routes ("i.m." or "i.m. and i.n.") were detectable (Fig. 3). All control animals ($n=10$) developed high levels of NS3-specific and neutralizing antibody titers following challenge. A marked booster reaction of NS3-specific and neutralizing antibodies could be detected in particular in animals receiving only one i.m. dose of pseudovirions ("1x_im" group; Fig. 3).

After challenge infection with heterologous BVDV strain SE5508, all animals vaccinated twice remained healthy and did not exhibit any clinical sign of BVDV infection (data not shown). No leukopenia was observed for animals of the immunization groups "2x_im" and "2x_im_in" (Fig. 4). In addition, neither challenge virus shedding nor viremia could be detected in these animals (Fig. 5). In contrast, unvaccinated control animals developed clinical signs such as coughing and fever. Leukopenia, viremia and shedding of the challenge virus until day 10 post challenge infection were also detected (Figs. 4 and 5). Animals challenged 29 days after the first immunization (group "1x_im") were only partially protected, but virus shedding as well as leukopenia were reduced when compared to sham-vaccinated control animals (Figs. 4 and 5).

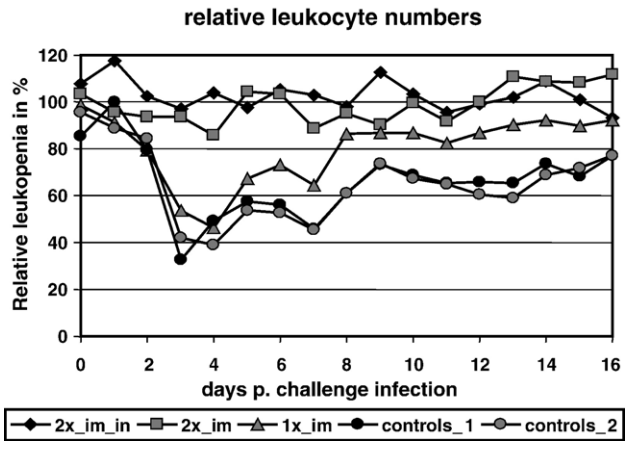

Fig. 4. Relative leukopenia (mean value before challenge infection = 100%) in the different treatment groups is shown as mean percentages. All control animals as well as the calves immunized once showed a marked leukopenia after challenge infection. In contrast, all calves immunized twice with packaged NCP7ΔC replicons were fully protected from BVDV-induced leukopenia.

Discussion

In this study, BVDV mutants unable to express functional structural proteins were constructed (NCP7ΔC, NCP7ΔE^{RNS}, NCP7ΔE1) and characterized. Transfection of *in vitro*-transcribed RNA of all constructs resulted in efficient replication of RNA that was comparable to that of parental NCP7 RNA, and expression of BVDV proteins was readily detected. However, no infectious virus could be recovered after transfection of cells permissive for BVDV replication, even after serial passages using for example the highly susceptible KOP-R cell line. In contrast, transfection of full-length NCP7 RNA led to production of infectious progeny virus and titers of approximately 10^3 IU/ml were reached at 24 h p.t. These results are in accordance with earlier studies using NCP7ΔE2 replicons in which no infectious virus was produced upon transfection of non-complementing cells (Harada et al., 2000; Reimann et al., 2003). Naturally occurring subgenomic pestivirus RNAs with deletions within the genome regions encoding the structural proteins and non-structural proteins N^{pro}, p7 and NS2 have been described for BVDV and CSFV isolates (Kupfermann et al., 1996; Meyers and Thiel, 1995). These so-called defective interfering particles (DIs) are able to replicate autonomously in susceptible cells but fail to produce infectious virions (Behrens et al., 1998; Moser et al., 1996). It is also known that neither the non-structural proteins N^{pro}, p7 and NS2 nor any of the structural proteins are essential for RNA replication of pestiviruses

Days p. inf.		1	2	3	4	5	6	7	8	9	10	11
2x_im_in	No.173	0a/0b	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
	No.537	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
	No.554	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
	No.758	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
	No.977	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
2x_im	No.1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
	No.2	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
	No.3	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
	No.4	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
	No.5	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
1x_im	No.11	0/0	0/0	0/0	1/1	1/1	0/0	0/0	0/0	0/0	0/0	0/0
	No.12	0/0	0/0	0/0	0/0	0/0	1/0	0/0	0/0	0/0	0/0	0/0
	No.13	0/0	0/0	0/0	2/0	2/1	0/0	0/0	0/0	0/0	0/0	0/0
	No.14	0/0	0/0	0/0	2/0	1/1	1/1	0/0	0/0	0/0	0/0	0/0
	No.15	0/0	0/0	0/0	0/0	1/0	1/0	0/0	0/0	0/0	0/0	0/0
Controls1	No. 97	0/0	0/0	2/2	2/1	2/2	2/1	2/2	0/2	0/1	0/1	0/0
	No. 99	0/0	0/0	2/0	2/2	2/2	2/2	0/2	1/2	0/2	0/0	0/0
	No.172	0/0	0/0	0/0	1/-	2/-	2/2	1/2	2/2	0/2	0/0	0/0
	No.610	0/0	0/0	1/2	2/2	2/1	2/1	2/2	2/0	1/1	0/0	0/0
	No.611	0/1	2/2	1/1	1/2	2/2	2/2	2/2	0/1	0/0	0/0	0/0
Controls2	No. 6	0/0	0/0	1/0	2/0	1/0	2/0	1/0	2/1	0/0	0/0	0/0
	No. 7	0/0	0/0	2/0	1/0	2/1	2/2	1/2	1/0	0/0	0/0	0/0
	No. 8	0/0	0/0	0/0	1/0	2/0	2/2	1/1	0/0	0/0	0/0	0/0
	No. 9	0/0	0/0	0/0	2/0	1/0	0/1	0/0	0/0	0/0	0/0	0/0
	No.10	0/0	0/0	0/0	1/0	2/1	2/1	2/0	1/2	1/0	1/0	0/0

a: blood leukocytes, b: nasal swabs (2= both inoculations positive, 1 = 1 of 2 inoculations positive, 0 = negative)

Fig. 5. Viremia and virus shedding after challenge infection. Peripheral blood leukocytes and nasal swabs were inoculated in duplicate into highly susceptible KOP-R cell cultures and stained for BVDV antigen after 4 to 5 days. Results were scored according to the number of positive inoculations (2=both inoculations BVDV-positive, 1=1 of 2 inoculations BVDV-positive, 0=no BVDV isolation).

(Behrens et al., 1998, Meyers et al., 1996; Moser et al., 1996), but infectious virus could be generated by complementing the missing proteins and packaging the defective genomes with a helper virus (Kupfermann et al., 1996). Concerning the four structural proteins of BVDV, E2-deleted replicons have been reported so far (Harada et al., 2000; Reimann et al., 2003), and it was demonstrated that E2 is essential for production and release of infectious virus progeny. Therefore, our experiments also prove that all structural proteins of BVDV are essential for the generation of infectious virus.

Trans-complementation of flavivirus proteins has been reported for example in the case of YFV and KUN non-structural proteins NS1, NS3 and NS5 (Khromykh et al., 1998; Lindenbach and Rice, 1997), for CSFV-E^{RNS} and -E2 (Frey et al., 2006; Widjojoatmodjo et al., 2000), and BVDV-E2 and -E2-p7 (Harada et al., 2000; Reimann et al., 2003) using helper cell lines or viral vectors expressing the deleted proteins. In the present study, the newly generated replicons NCP7ΔC, NCP 7ΔE^{RNS} and NCP7ΔC were efficiently *trans*-complemented and packaged into virions using the helper cell line PT_805. It is important to emphasize that we did not observe any recombination or reversion during numerous *trans*-complementation experiments and passages on the PT_805 cells, likely because we were utilizing a synthetic, codon-optimized version of the BVDV structural gene region (Reimann et al., 2003), which greatly reduces the potential for homologous recombination events. In addition, the PT_805 cells were the most efficient packaging system available. Nevertheless, novel techniques for the generation of helper cell lines, like VEEV-replicons (Mason et al., 2006) will be also tested in the future for the complementation of BVDV-replicons.

In the first passage, maximum virus titers of the *trans*-complemented pseudovirions 'NCP7ΔC_trans', 'NCP7ΔE1_trans' and 'NCP7ΔE^{RNS}_trans' ranged between $10^{5.5}$ to $10^{6.125}$ IU/ml at 24 h p.t. Infection of non-complementing cells with the pseudovirions resulted in efficient RNA replication and protein expression without the generation of infectious virus particles. After passaging on non-complementing KOP-R cells, no recombination or reversion resulting in infectious virions could be detected either, similar to the situation in PT_805 cells. Interestingly, a markedly reduced susceptibility of PT_805 cells to infection with all *trans*-complemented replicons engineered here was observed (data not shown), which is in good agreement with our earlier findings using NCP7ΔE2_trans pseudovirions (Reimann et al., 2003). This phenomenon of viral interference was also described for other pestiviruses (Harada et al., 2000; Mittelholzer et al., 1997), but, nonetheless, is not well understood. Recent studies of Lee et al. (2005) suggest a dual mechanism of pestivirus interference, one at the level of viral entry caused by glycoprotein E2 and a second at the level of viral RNA replication where the viral and cellular components remain to be identified. Therefore, the newly constructed replicons as well as the helper cell line PT_805 will provide a useful basis for further studies on pestivirus interference. In addition, the direct

generation of virus like particles (VLPs) by the capsid-deleted replicon construct in non-complementing cells, as demonstrated for TBEV (Mandl, 2004), can be investigated in the future. However, in a first preliminary electron microscopy analysis, no BVDV-VLPs were detected (data not shown).

The generated BVDV pseudovirions were also characterized *in vivo* as a novel approach to control BVDV infections by vaccination. In spite of the wide use of vaccination against BVDV infections in comprehensive control programs, serious concerns exist regarding the safety and/or efficacy of conventional modified live virus or inactivated vaccines (Becher et al., 2001; Bolin and Ridpath, 1995b; Fernelius et al., 1971; Howard et al., 1994; Thierauf, 1993). While some of the modified live virus vaccines were shown to induce a robust immunity similar to that induced by natural BVDV infection, inactivated BVDV vaccines are not universally accepted to be sufficiently efficacious (Beer et al., 2000; Beer and Wolf, 2003; Bolin and Ridpath, 1995b). Because of the serious risks of an application of infectious BVDV to pregnant animals, the development of new efficacious and safe vaccines is of paramount importance. The main aim of an immunization against BVDV infection is protection against virus shedding, viremia, and subsequent transplacental infection of a fetus. In this context, cellular immunity might play a crucial role for a protective BVDV immunity (Beer et al., 1997; Collen and Morrison, 2000), which could also explain the problems of inactivated vaccines to fully protect against BVDV viremia.

So-called defective in second cycle (DISC) viruses could be the basis for such an improved BVD/MD vaccine, and pestivirus pseudovirions, such as the various mutant replicons described here, might be a very promising prototype of a safe 'live' vaccine. In experiments using E^{RNS}- or E2-deleted CSFV replicons, however, only partial protection against subsequent challenge infection could be demonstrated, and protection was shown to be restricted to intradermal immunization (van Gennip et al., 2000; van Gennip et al., 2002).

In our *in vivo* studies, we concentrated on the capsid-deleted replicon for the immunization of cattle since the most immunogenic envelope proteins, E^{RNS} and E2, are still expressed by this construct. In both immunization and challenge trials with pseudovirions lacking capsid sequences, specific antibody responses could be detected even after a single intramuscular immunization. Besides neutralizing antibodies, NS3-specific antibodies were detected, which are sensitive indicators of pestiviral RNA replication and protein expression, and both types of antibodies were efficiently boostered after a second immunization. In addition, two i.m. applications were as efficient as two combined 'i.m.+i.n.' applications. It became clear, however, that only animals immunized twice (i.m. or i.m.+i.n.) were completely protected against heterologous BVDV challenge infection, whereas the one-dose immunization reduced clinical signs and virus shedding, but was unable to induce sterile immunity. Most notably, the observed immune response against NCP7ΔC pseudovirions was reminiscent of both inactivated pestivirus vaccines as reflected by low titers after the first immunization, partial protection against viremia and effective booster vaccination (Beer and Wolf, 2003), and attenuated live vaccines that are characterized by an early NS3-specific antibody response and a sterile immunity (Beer and Wolf, 2003; Moennig et al., 2005). Therefore, the BVDV standard challenge model with a BVDV type 1 strain not only simulates a field infection but also allows a clear graduation between the groups, even without inducing severe clinical signs. As a further consequence of the results of the immunization study, additional vaccination and booster schemes are conceivable, e.g. using inactivated vaccines or recombinant vectors for booster immunizations.

As an important safety feature and in contrast to packaged alphavirus replicons (Bredenbeek et al., 1993; Frolov et al., 1996; Weiss and Schlesinger, 1991), no revertant, pseudorevertant or recombined BVDV replicons were detected in our study. As briefly explained earlier, we suggest that RNA recombination is extremely rare or even impossible in the reported BVDV replicon packaging system that uses a synthetic open reading frame encoding all structural proteins (Reimann et al., 2003). While recombination in the non-structural region with a single template switch was demonstrated for pestiviruses (Becher et al., 2001), the double template switch needed for a recombination event in the system presented here has not been reported and is considered highly unlikely.

In conclusion, we describe here the construction and successful testing of a new pestivirus vaccine prototype, which combines the safety aspects of inactivated vaccines with the efficacy of an attenuated, modified live vaccine. In contrast to other flavivirus pseudovirion models, protective immunity could be induced in a natural virus–host system using intramuscular immunization. Furthermore, the reported type of vaccine virus could also serve as a model for similar capsid-deleted replicons for related viruses, such as HCV. Since efficiently replicating systems for HCV are now available (Lindenbach et al., 2005), our data suggest that the construction and testing of HCV pseudovirions for immunization might be a goal that is worthwhile pursuing.

Material and methods

Cells and viruses

PT cells (RIE11, CCLV), a permanent ovine cell line, KOP-R cells (Rie244, CCLV), a diploid bovine esophageal cell line, and PT_805 cells (Reimann et al., 2003), a cell line expressing the structural proteins C, E^{RNS}, E1 and E2 of BVDV, were obtained from the collection of cell lines in veterinary medicine at the Federal Research Institute for Animal Health, Insel Riems (CCLV). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% BVDV-free fetal bovine serum (FBS). BVDV strain NCP7 was rescued from infectious cDNA clone pA/BVDV/Ins^- (Meyers et al., 1996) after transfection of PT cells and passaging in KOP-R cells. Virulent BVDV type 1 challenge virus strain SE5508 (Wolfmeyer et al., 1997) was propagated in KOP-R cells. *Trans*-complemented viruses NCP7ΔC_trans, NCP7ΔE1_trans, and NCP7ΔE^{RNS}_trans are pseudovirions produced after transfection of the different replicons into the helper cell line PT_805.

Monoclonal antibodies, ELISA, IF analysis and neutralization assay

For the detection of BVDV proteins, monoclonal antibodies (Mab) *WB210* (anti-E^{RNS}, CVL, Weybridge), CA3 (anti-E2, TiHo, Hannover, Germany), and *C16* (anti-NS3, TiHo, Hannover, Germany) were used (Edwards et al., 1988, 1991; Peters et al., 1986). Standard immunofluorescence (IF) analysis using a fluorescence-activated cell sorter (FACS, Becton Dickinson) or a fluorescence microscope (Olympus) were performed as previously described (Beer et al., 1997; Grummer et al., 2001). For IF analysis using a fluorescence microscope, cell cultures were fixed with 3% paraformaldehyde (PFA) and permeabilized using 0.01% digitonin or fixed/permeabilized using 80% acetone (for E2 staining). After washing with phosphate-buffered saline (PBS), cells were incubated with the working dilution of the appropriate Mab for 15 min. After two additional washing steps with PBS, cells were incubated with an $Alexa^{488}$ conjugate (Invitrogen) for 15 min, finally washed and analyzed by fluorescence microscopy.

Standard neutralizing assays using inactivated sera were performed as described previously (Beer et al., 1997; Reimann et al., 2004). In addition, serum or plasma samples were tested for the presence of antibodies against BVDV with the commercial ELISA assays HerdCheck BVDV AB (IDEXX), BVD Virus III Chekit® (Bommeli Diagnostics) and Ceditest® BVDV (Cedi Diagnostics B.V.) according to the manufacturers' instructions.

In vitro-transcription and electroporation

In vitro-transcription of linearized, full-length cDNA constructs and replicons was performed by T7 RiboMax™ Large Scale RNA Production System (Promega) according to the manufacturer's instructions. The amount of RNA was estimated by ethidium bromide staining after agarose gel electrophoresis. For transfections, 1×10^7 PT_805 or KOP-R cells were detached using a trypsin solution, washed with PBS, mixed with 1 to 5 μg of RNA synthesized *in vitro*, and electroporated using an *EasyjecT Plus* (EquiBio) transfection unit (two pulses at 850 V, 25 μF, 156 Ω).

Polymerase chain reaction (PCR) and sequencing

For PCR, a PTC-200 thermal cycler (MJ Research, Inc.) was used. DNA based amplification was done using the Expand High Fidelity PCR System (Roche Molecular Biochemicals). For RT–PCR, total RNA of virus-infected cells was extracted using the TRIZOL reagent (Gibco-Life Technologies). RT–PCR was performed by using the One-step RT–PCR Kit (Qiagen), and PCR products were directly sequenced.

Sequencing was carried out using a Thermo Sequenase Cycle Sequencing Kit (Amersham Biosciences). Nucleotide sequences were read with a LI-COR automatic sequencer (MWG Biotech) and analyzed using GCG version 11.1 (Accelrys Inc., San Diego, CA). Primers used for PCR or sequencing were custom synthesized (MWG-Biotech).

Plasmid constructs

Mutant BVDV clones are depicted in Fig. 1. They were constructed based on a full-length cDNA clone, pA/BVDV/Ins^-, harboring the non-cytopathogenic BVDV strain NCP7 genome (Meyers et al., 1996). The specified nucleotide positions correspond to the BVDV NCP7 genome (Accession No. BVU43679; Meyers et al., 1996). Restriction enzyme digestion and cloning procedures were performed according to standard protocols. PCR fragments used for the construction of the various replicons were derived from a NCP7 DNA fragment with a mutated *Kpn*I site at position 2447, which was subcloned into plasmid pUC18 (Clontech). We constructed several BVDV NCP7 mutants with in-frame deletions of C, E^{RNS}, or E1. Sequences known to be important for correct translocation of viral proteins or for signalase recognition (Rümenapf et al., 1991) were preserved. The capsid-deleted replicon NCP7ΔC contained a deletion of amino acids 201–242 compared to the parental NCP7 ORF. The N-terminal 32 amino acids and the 27 C-terminal amino acids of the C protein, which are essential for signalase recognition, translocation of the envelope proteins into the endoplasmatic reticulum (ER), and further processing of the E^{RNS}E1E2 polyprotein (Rümenapf et al., 1991, 1993), were retained. The construct NCP7ΔE^{RNS} was designed with a restricted deletion within the central region of E^{RNS} (amino acids 321 to 475), because the N- and C-terminal E^{RNS} sequences also contain important signal sequences for cellular proteases (Donis et al., 1988; Rümenapf et al., 1991; Thiel et al., 1991). The envelope protein E1, which corresponds to amino acids 497 to 692, harbors two hydrophobic stretches at the C-terminus, which act as a stop-transfer signal and a signal sequence to induce the translocation of the envelope protein E2 into the ER lumen. The hydrophobic sequences also form an anchor for E1. Therefore, only the N-terminal sequence region was deleted (amino acids 498 to 654) and 37 C-terminal amino acids were maintained in the E1-deleted replicon.

Complementation experiments

PT_805 helper cells were transfected with *in vitro*-transcribed BVDV replicon RNA by electroporation as described before (Reimann et al., 2003). Cell culture supernatants were collected 24 h to 48 h *post transfection* (p.t.), clarified by centrifugation (10,000×*g*, 5 min), and titrated using KOP-R cells. At the day of collection, replication of BVDV was monitored by IF staining.

Pseudovirion titers were determined as infectious units (IU) as described previously (Reimann et al., 2003). Cell culture supernatants were titrated in triplicate in $\log_{10}$ steps and 1 ml was inoculated onto KOP-R cells seeded in 24-well plates. After 12 to 24 h of incubation at 37 °C, cells were washed, detached with a trypsin solution, and counted. An aliquot of the cells was stained by IF using a BVDV NS3-specific Mab and analyzed by flow cytometry. All transfection and passaging experiments were repeated at least 10 times for each of the three replicon constructs (Table 1).

Animal experiments

Four- to six-month-old, healthy, BVD antibody- and virus-free calves (Simmental breed) were immunized with packaged NCP7ΔC replicons using different protocols. In two independent experiments, three different immunization groups of 5 calves each were investigated: The first group (1x_im) was immunized once by the intramuscular (i.m.) route at day 0. The second group (2x_im) received two i.m. immunizations at days 0 and 29, and the third group (2x_im_in) was immunized i.m. and intranasally (i.n.) at days 0 and 29, respectively. Each animal received 2 ml of a preparation containing a pseudovirion titer between $10^{5.625}$ and $10^{6.125}$ IU/ml. Five mock-inoculated animals were used as controls in each experiment. All animals were tested for clinical signs, virus shedding, and viremia following immunization and challenge infection. Intranasal challenge infection was performed 28 days after the last immunization using 2 ml of a preparation with a titer of 10^6 $TCID_{50}$/ml of the virulent, heterologous BVDV strain SE5508.

After immunization and challenge infection, virus isolation was performed in duplicates on KOP-R cells with blood samples and nasal swabs using co-cultivation of 1×10^6 blood leukocytes or 100 μl of nasal swab supernatants. After an incubation period of 4 to 5 days, the cells were analyzed for NS3 expression by IF.

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